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Appraisal on the wound healing and anti-inflammatory activities of the essential oils obtained from the cones and needles of *Pinus* species by *in vivo* and *in vitro* experimental models

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ABSTRACT

Ethnopharmacological relevance: According to ethnobotanical data, *Pinus species* have been used against rheumatic pain and for wound healing in Turkish folk medicine.

Essential oils from the cones and needles of five different *Pinus* species (*Pinus brutia* Ten., *Pinus halepensis* Mill., *Pinus nigra* Arn., *Pinus pinea* L. and *Pinus sylvestris* L.) were evaluated for their *in vivo* wound healing and anti-inflammatory activities.

Materials and methods: In vivo wound healing activity of the ointments prepared from essential oils was evaluated by linear incision and circular excision experimental wound models subsequently histopathological analysis and hydroxyproline content. Furthermore, the essential oils were screened for anti-hyaluronidase activity. Additionally anti-inflammatory activity was assessed by using the method of Whittle, which is based on the inhibition of acetic acid-induced increase in capillary permeability.

Results: The essential oils obtained from the cones of *Pinus pinea* and *Pinus halepensis* demonstrated the highest effects on the wound healing activity models. On the other hand, the rest of the essential oils did not show any significant wound healing and anti-inflammatory activities.

Conclusion: The experimental study revealed that essential oils obtained from the cones of *Pinus pinea* and *Pinus halepensis* display remarkable wound healing activity.

1. Introduction

Pinus genus belongs to the family Pinaceae, which is found in most of the Northern Hemisphere (Kurose et al., 2007). Turkey is rich in coniferous woods which grow in different regions of the country (Hafızoglu and Usta, 2005; Tumen et al., 2010). Pine oils are used as flavoring additives for food and beverages, fragrances in cosmetics and scenting agents in household products (Ustun et al., 2006; Sezik et al., 2010). In folk medicine, various parts of *Pinus* species (bark, needle, cone and resin) have been used for rheumatism or as anti-inflammatory, antioxidant and antiseptic (Baytop, 2001). In Turkish folk medicine, resin of *Pinus nigra* and *Pinus sylvestris* is used externally for wound healing. Decoction is used for common colds and against stomachache. Tar of *Pinus sylvestris* is mixed with that of *Juniperus* and leaves of *Sambucus ebulus* and boiled together. Rheumatic pains are treated by bathing the patient in this extract (Fujita et al., 1995). Fruit decoction of *Pinus brutia* is used internally to treat diarrhoea (Yesilada et al., 1993). For the treatment of peptic ulcers, resin of *Pinus brutia* is taken orally every other day, alternately with the resin of *Pistacia terebinthus* (Yesilada et al., 1995).

Essential oils obtained from aromatic and medicinal plants generally possess biological effects such as antibacterial, antifungal and antioxidant activities (Tumen et al., 2010). Essential oil constituents of the cones of the family Pinaceae possess antioxidant, analgesic and anti-HIV activities (Sakagami et al., 1991; Gülçin et al., 2003). Phytochemical analysis revealed that terpenoids, steroids, procyanidins and flavonoids are the ingredients of *Pinus* extracts (Sakar et al., 1991; Tanaka et al., 1999; Lantto et al., 2009). The compounds exerted antibacterial and antifungal activities against acnes. Resin acids such as isopimaric and abietic acids extracted from the cones of *Pinus nigra* displayed antibacterial activity (Smith et al., 2005).

In this study we aimed to evaluate the wound healing and anti-inflammatory activities of the essential oils from the cones and needles of five different *Pinus* species (*Pinus brutia* Ten., *Pinus halepensis* Mill., *Pinus nigra* Arn., *Pinus pinea* L. and *Pinus sylvestris* L.).

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Table 1
Sampling site, climate zone, date and altitude of the analyzed tree species.

Species	Sampling site	Local name	Collection date	Altitude (m)	Herbarium num.
Pinus brutia Ten.	Yenice- Northwest Turkey	Kızılçam	June, 2010	810	GUE 2979
Pinus halepensis Mill.	Adana-South Turkey	Halep Çamı	October, 2010	1100	GUE 2980
Pinus nigra Arn.	Bartın-Northwest Turkey	Karaçam	September, 2010	740	GUE 2981
Pinus pinea L.	Bartın-Northwest Turkey	Fıstık Çamı	March, 2010	600	GUE 2982
Pinus sylvestris L.	Bartın-Northwest Turkey	Sarıçam	September, 2010	700	GUE 2983

Table 2
Effects of the essential oils from *Pinus brutia, Pinus halepensis, Pinus nigra, Pinus pinea* and *Pinus sylvestris* on linear incision wound model.

Material	Parts used	Statistical Mean ± S.E.M.	(Tensile strength %)
Vehicle		9.54 ± 2.16	5.3
Negative Control		9.06 ± 2.12	–
Pinus brutia	Cones	9.80 ± 2.19	2.7
	Needles	9.18 ± 1.96	–
Pinus halepensis	Cones	11.81 ± 1.27	**23.8***
	Needles	10.96 ± 1.83	14.9
Pinus nigra	Cones	11.41 ± 2.11	19.6
	Needles	10.99 ± 1.26	15.2
Pinus pinea	Cones	13.25 ± 1.84	**38.9****
	Needles	11.06 ± 1.35	15.9
Pinus sylvestris	Cones	9.21 ± 2.31	–
	Needles	9.72 ± 1.71	1.9
Madecassol®		14.79 ± 1.23	**55.0*****

Percentage of tensile strength values: Vehicle group was compared to Negative control group; The extracts and the reference material were compared to vehicle group.
S.E.M.: Standard error mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

2. Materials and methods

2.1. Plant material

The cones and needles parts of different pine tree species (*Pinus halepensis* Mill., *Pinus pinea* L., *Pinus sylvestris* L., *Pinus nigra* Arn. and *Pinus brutia* Ten.) used in this study were collected directly different parts of the trees from the region of Adana, Bartin and Yenice, respectively. The voucher specimens of the plants were deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey. The pine species were collected according to the conventional method (Davis et al., 1965; Dığrak et al., 1999). 2 kg of cones and needles were collected for each species from their growth sites just at the time of maturity. The collected parts were ground using a blender (Dığrak et al., 1999). Species names, sampling site, collection date, climate zone, altitude and herbarium number of all specimens are listed in Table 1 (Tumen et al., 2010).

2.2. Essential oils

The essential oils of each sample were obtained by hydrodistillation with a Clevenger apparatus using 1000 g (partially crushed) of fresh cones and needles. The obtained oils were collected for 6 h and subsequently dried over anhydrous sodium sulphate and under refrigeration in a sealed vial until analyzed and tested (Tunalier et al., 2002).

2.3. Biological activity tests

2.3.1. Animals

Male, Sprague–Dawley rats (160–180 g) and Swiss albino mice (20–25 g) were purchased from the animal breeding laboratory of Saki Yenilli (Ankara, Turkey).

The animals were left for 3 days at room conditions for acclimatization. They were maintained on standard pellet diet and water *ad libitum* throughout the experiment. A minimum of six animals were used in each group. Throughout the experiments, animals were processed according to the suggested European ethical guidelines for the care of laboratory animals (Gazi University Ethical Council Project Number: G.U.ET-08.037).

2.3.2. Preparation of test samples for bioassay

Incision and excision wound models were used to evaluate the wound healing activity. For the *in vivo* wound models, test samples were prepared in an ointment base (vehicle) consisting of glycol stearate, 1,2 propylene glycol, liquid paraffin (3:6:1) in 1% concentration. 0.5 g of each test ointment was applied topically on the wounded site immediately after wound was created by a surgical blade.

The animals of the vehicle group were treated with the ointment base only, whereas the animals of the reference drug group were treated with 0.5 g of Madecassol® (Bayer, 00001199). Madecassol contains 1% extract of *Centella asiatica*.

For the assessment of anti-inflammatory activity, test samples were given orally to test animals after suspending in a mixture of distilled H_2O and 1% Tween 80. The control group animals received the same experimental handling as those of the test groups except the drug treatment was replaced with appropriate volumes of dosing vehicle. Indomethacin (10 mg/kg) in 1% Tween 80 was used as reference drug.

2.3.3. Wound healing activity

2.3.3.1. Linear incision wound model. Animals, seven rats in each group, were anaesthetized with 0.15 cc Ketasol® (Richterpharma). The hairs on the dorsal part of the rats were shaved and the skin was cleaned with 70% alcohol. Two 5 cm-length linear-paravertebral incisions were made with a sterile blade through the shaved skin at

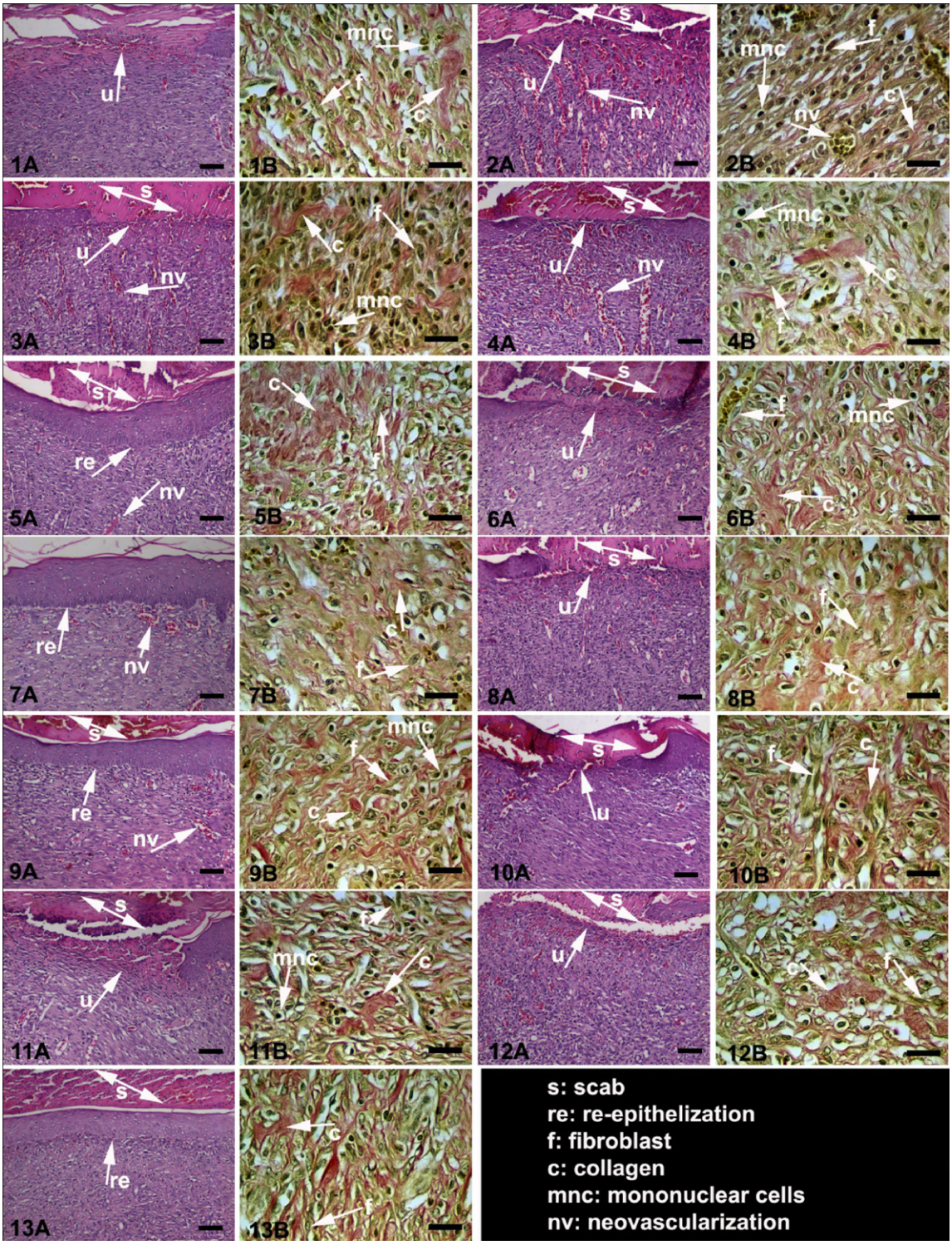

Fig. 1. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, oils and reference ointment Madecassol® administered animals. Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in A, and the dermis stained with Van Gieson (VG) in B. The original magnification was 100× and the scale bars represent 120 μm for figures in A, and the original magnification was 400× and the scale bars represent 40 μm for B. Data are representative of 6 animal per group. (1) Vehicle group, 10 day old wound tissue treated with only vehicle, (2) Negative Control group, 10 day old wound tissue, untreated group, (3) *Pinus brutia* cones group, 10 day old wound tissue treated with the essential oil of *Pinus brutia* cones, (4) *Pinus brutia* needles group, 10 day old wound tissue treated with the essential oil of *Pinus brutia* needles, (5) *Pinus halepensis* cones group, 10 day old wound tissue treated with essential oil of *Pinus halepensis* cones, (6) *Pinus halepensis* needles group, 10 day old wound tissue treated with essential oil of *Pinus halepensis* needles, (7) *Pinus nigra* cones group, 10 day old wound tissue treated with essential oil of *Pinus nigra* cones, (8) *Pinus nigra* needles group, 10 day old wound tissue treated with essential oil of *Pinus nigra* needles, (9) *Pinus pinea* cones group, 10 day old wound tissue treated with essential oil of *Pinus pinea* cones, (10) *Pinus pinea* needles group, 10 day old wound tissue treated with essential oil of *Pinus pinea* needles, (11) *Pinus sylvestris* cones group, 10 day old wound tissue treated with essential oil of *Pinus sylvestris* cones, (12) *Pinus sylvestris* needles group, 10 day old wound tissue treated with essential oil of *Pinus sylvestris* needles, (13) Reference group, 10 day old wound tissue treated with Madecassol®.

Table 3
Effects of the essential oils from *Pinus brutia*, *Pinus halepensis*, *Pinus nigra*, *Pinus pinea* and *Pinus sylvestris* on circular excision wound model.

Material	Parts used	Wound area ± S.E.M. (Contraction %)						
		0	2	4	6	8	10	12
Vehicle		19.40 ± 2.29	17.65 ± 2.17 (4.59)	16.05 ± 2.10 (9.68)	14.22 ± 1.68 (4.76)	10.06 ± 1.63 (8.71)	6.24 ± 1.46 (13.45)	2.82 ± 0.63 (9.90)
Negative Control		19.51 ± 2.18	18.50 ± 2.06	17.77 ± 2.25	14.93 ± 1.96	11.02 ± 1.68	7.21 ± 1.19	3.13 ± 1.28
Pinus brutia	Cones	19.46 ± 2.28	17.03 ± 1.91 (3.51)	16.21 ± 2.26–	13.96 ± 1.73 (1.83)	9.57 ± 1.61 (4.87)	6.07 ± 1.18 (2.72)	2.62 ± 1.14 (7.09)
	Needles	19.33 ± 2.01	17.58 ± 1.82 (0.39)	16.35 ± 2.20–	14.02 ± 1.68 (1.41)	9.73 ± 1.79 (3.28)	5.98 ± 1.22 (4.17)	2.99 ± 1.05–
Pinus halepensis	Cones	19.43 ± 2.09	16.26 ± 1.82 (7.88)	15.01 ± 1.84 (6.48)	13.33 ± 1.19 (6.26)	8.59 ± 1.24 (14.61)	5.24 ± 1.26 (16.03)	1.69 ± 0.30 **(40.07)****
	Needles	19.47 ± 2.54	17.15 ± 2.17 (2.83)	15.41 ± 1.87 (6.23)	13.10 ± 1.14 (7.88)	9.24 ± 1.65 (8.15)	6.05 ± 1.50 (3.04)	2.35 ± 0.44 (16.67)
Pinus nigra	Cones	20.10 ± 2.36	18.10 ± 2.24–	15.85 ± 1.98 (1.25)	14.36 ± 1.72–	9.99 ± 1.59 (0.69)	7.22 ± 1.37–	2.41 ± 0.68 (14.54)
	Needles	19.26 ± 2.19	16.81 ± 1.95 (4.76)	15.26 ± 1.91 (4.92)	12.15 ± 1.21 (14.56)	8.25 ± 1.37 (17.99)	5.09 ± 1.31 (18.43)	2.37 ± 0.36 (15.96)
Pinus pinea	Cones	19.96 ± 2.93	15.62 ± 1.75 (11.50)	14.73 ± 1.95 (8.22)	13.19 ± 1.41 (7.24)	8.22 ± 1.28 (18.29)	4.91 ± 1.13 (21.31)	1.28 ± 0.12 **(54.61)****
	Needles	19.49 ± 2.36	16.13 ± 2.04 (8.61)	15.01 ± 1.80 (6.48)	13.84 ± 1.30 (2.67)	9.01 ± 1.31 (10.44)	4.99 ± 0.94 (20.03)	2.25 ± 0.18 (20.21)
Pinus sylvestris	Cones	19.68 ± 2.45	17.84 ± 2.10–	16.25 ± 2.41–	14.13 ± 1.69 (0.63)	9.84 ± 1.52 (2.19)	6.43 ± 1.85–	3.18 ± 0.72–
	Needles	19.51 ± 2.30	17.04 ± 1.86 (3.46)	16.17 ± 2.13–	14.38 ± 1.34–	9.71 ± 1.63 (3.48)	6.37 ± 1.04–	3.03 ± 0.57–
Madecassol®		19.52 ± 2.13	15.09 ± 1.37 (14.50)	12.01 ± 1.71 (25.17)	8.82 ± 1.17 **(37.97)***	5.01 ± 0.96 **(50.20)****	1.85 ± 0.13 **(70.35)*****	0.00 ± 0.00 **(100.00)*****

Percentage of contraction values: vehicle group was compared to negative control group; the extracts and the reference material were compared to vehicle group.
S.E.M.: Standard error mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 4
Effect of topical treatment of test ointments for 7 days on hydroxyproline content.

Material	Parts used	Hydroxyproline (μg/mg) ± S.E.M.
Vehicle		21.4 ± 2.69
Negative control		20.3 ± 2.49
Pinus brutia	Cones	25.9 ± 2.63
	Needles	27.2 ± 2.14
Pinus halepensis	Cones	**38.2 ± 3.10***
	Needles	21.5 ± 2.07
Pinus nigra	Cones	27.8 ± 3.14
	Needles	19.9 ± 2.23
Pinus pinea	Cones	**47.1 ± 3.74****
	Needles	30.6 ± 2.98
Pinus sylvestris	Cones	19.2 ± 3.16
	Needles	17.54 ± 2.38
Madecassol®		**78.4 ± 2.51****

S.E.M.: Standard error mean
* $p < 0.05$.
** $p < 0.01$.

the distance of 1.5 cm from the dorsal midline on each side. Three surgical sutures were placed each 1 cm apart.

The ointments prepared with test samples, the reference drug (Madecassol®) or ointment base [glycol stearate: propylene glycol: liquid paraffin (3:6:1)] were topically applied on the dorsal wounds in each group of animals once daily throughout 9 days. On the 9th post wound day all the sutures were removed and on day 10 all the animals were killed under ether anaesthesia. Tensile strength of previously wounded and treated skin was measured by using a tensiometer (Zwick/Roell Z0.5, Germany) (Suguna et al., 2002; Lodhi et al., 2006; Küpeli Akkol et al., 2011).

2.3.3.2. Circular excision wound model. This model was used to monitor wound contraction and wound closure time. Each group of animals (seven animals in each) was anaesthetized by 0.01 cc Ketasol® (Richterpharma). The back hairs of the mice were depilated by shaving. The circular wound was created on the dorsal interscapular region of each animal by excising the skin with a 5 mm biopsy punch (Nopa instruments, Germany); wounds were left open (Tramontina et al., 2002). Test samples, the reference drug (Madecassol®, Bayer) and the vehicle ointments were applied topically once a day till the wound was completely healed. The progressive changes in wound area were monitored by a camera (Fuji, S20 Pro, Japan) every other day. Later on, wound area was evaluated by using AutoCAD program. Wound contraction was calculated as percentage of the reduction in wounded area. A specimen sample of tissue was isolated from the healed skin of each group of mice for the histopathological examination (Süntar et al., 2011).

2.3.3.3. Hydroxyproline estimation. Tissues were dried in hot air oven at 60–70 °C till consistent weight was achieved. Afterwards, samples were hydrolyzed with 6 N HCl for 4 h at 130 °C. The hydrolyzed samples were adjusted to pH 7 and subjected to chloramin T oxidation. The coloured adduct formed with Ehrlich reagent at 60 °C was read at 557 nm. Standard hydroxyproline was also run and values reported as μg/mg dry weight of tissue (Rasik et al., 1999).

2.3.3.4. Histopathology. The skin specimens from each group were collected at the end of the experiment (on day 12). Samples were fixed in 10% buffered formalin, processed and blocked with paraffin and then sectioned into 5 micrometer sections and stained with hematoxylin & eosin (HE) and Van Gieson (VG) stains. The tissues were examined by light microscope (Olympus CX41

Table 5
Wound healing processes and healing phases of the vehicle, negative control, oils and Madecassol® administered animals.

Groups	Parts used	Wound healing processes								Healing phases		
		S	U	RE	FP	CD	MNC	PMN	NV	I	P	R
Vehicle		++/+++	++	−/+	++/+++	++	+/++	++	++	++	++/+++	−/+
Negative Control		++/+++	++/+++	−/+	++/+++	++	++	++/+++	++	++/+++	++/+++	−/+
Pinus brutia	Cones	++/+++	+/++	−/+	++/+++	++	++	++	++	++	++/+++	−/+
	Needles	++/+++	++	−/+	++/+++	+/++	++	+	+/++	++	++	−/+
Pinus halepensis	Cones	++	–	++	++	+++	+/++	+	++	+/++	++	++
	Needles	+++	++	+	+/++	+	+/++	++	++	+/++	++/+++	+
Pinus nigra	Cones	++/+++	–	++	++/+++	++	+/++	+	++	+/++	++	++
	Needles	+++	++/+++	−/+	++/+++	++	++	+/++	+/++	+++	+++	+
Pinus pinea	Cones	+	–	++	++	+++	+/++	+	+/++	+/++	++	++
	Needles	++	+++	++	+/++	+/++	+/++	++/+++	++	++	+/++	−/+
Pinus sylvestris	Cones	++/+++	++	−/+	++/+++	++	++	++	++	++	++/+++	−/+
	Needles	++	++	–	++/+++	++	+/++	+++	+	+++	+++	+
Madecassol®		+/++	–	++/+++	+/++	+++	+	−/+	+	+	+/++	++/+++

HE and VG stained sections were scored as mild (+), moderate (++) and severe (+++) for epidermal and/or dermal re-modeling. S: Scab, U: Ulcus, RE: Re-epithelization, FP: Fibroblast proliferation, CD: Collagen depositions, MNC: Mononuclear cells, PMN: Polymorphonuclear cells, NV: Neovascularization, I: Inflammation phase, P: Proliferation phase, R: Re-modeling phase.

Table 6
Effect of various concentrations of *Pinus* essential oils on hyaluronidase inhibition.

Material	Parts used	Concentrations (mg/ml)	% Inhibition (Mean ± SD)
Pinus brutia	Cones	100	17.53 ± 0.92
		200	20.41 ± 0.76
	Needles	100	10.14 ± 1.13
		200	18.21 ± 0.71
Pinus halepensis	Cones	100	29.16 ± 0.86
		200	**35.44 ± 0.79***
	Needles	100	19.47 ± 0.84
		200	27.15 ± 0.95
Pinus nigra	Cones	100	23.65 ± 0.55
		200	28.54 ± 0.61
	Needles	100	25.24 ± 1.17
		200	30.28 ± 0.93
Pinus pinea	Cones	100	**68.42 ± 0.50****
		200	**76.40 ± 0.47****
	Needles	100	27.20 ± 0.81
		200	28.29 ± 0.66
Pinus sylvestris	Cones	100	20.80 ± 0.81
		200	25.55 ± 0.68
	Needles	100	15.46 ± 0.99
		200	16.37 ± 0.73

S.E.M.: standard error mean.
* $p < 0.05$.
** $p < 0.01$.

attached Kameram® Digital Image Analyze System) and graded as mild (+), moderate (++) and severe (+++) for epidermal or dermal re-modeling. Re-epithelization or ulcus in epidermis; fibroblast proliferation, mononuclear and/or polymorphonuclear cells, neovascularization and collagen depositions in dermis were analyzed to score the epidermal or dermal re-modeling. Van Gieson stained sections were analyzed for collagen deposition. At the end of the examination, all the wound healing processes were combined and staged for wound healing phases as inflammation, proliferation, and re-modeling in all groups.

2.3.3.5. Determination of hyaluronidase inhibition. For the evaluation of the hyaluronidase inhibitory activity, two different measurements were taken as given below.

Table 7
Effect of *Pinus* essential oils on hyaluronidase activity of *Naja naja* venom.

Material	Parts used	Venom:Oil	Hyaluronidase activity ($\times 10^{-3}$)[a]
Control			3.12 ± 0.15
Pinus brutia	Cones	1:0.5	2.78 ± 0.22
		1:1	2.15 ± 0.14
		1:1.5	2.09 ± 0.31
	Needles	1:0.5	2.47 ± 0.29
		1:1	2.39 ± 0.41
		1:1.5	2.21 ± 0.45
Pinus halepensis	Cones	1:0.5	2.11 ± 0.27
		1:1	1.74 ± 0.11
		1:1.5	0.98 ± 0.23
	Needles	1:0.5	3.24 ± 0.62
		1:1	2.74 ± 0.33
		1:1.5	2.46 ± 0.60
Pinus nigra	Cones	1:0.5	2.35 ± 0.29
		1:1	1.82 ± 0.14
		1:1.5	1.02 ± 0.25
	Needles	1:0.5	2.12 ± 0.16
		1:1	2.01 ± 0.09
		1:1.5	1.88 ± 0.28
Pinus pinea	Cones	1:0.5	2.05 ± 0.19
		1:1	0.92 ± 0.10
		1:1.5	0.54 ± 0.16
	Needles	1:0.5	2.79 ± 0.34
		1:1	1.98 ± 0.26
		1:1.5	1.74 ± 0.42
Pinus sylvestris	Cones	1:0.5	3.24 ± 0.18
		1:1	3.11 ± 0.28
		1:1.5	2.98 ± 0.44
	Needles	1:0.5	2.62 ± 0.47
		1:1	2.21 ± 0.20
		1:1.5	2.13 ± 0.17

Values presented as mean ± S.E.M.
[a] Activity is epressed in terms of N-acetyl glucosamine released in n mol/min/mg protein.

1. The inhibition of hyaluronidase was assessed by the measurement of the amount of *N*-acetylglucosamine released from sodium hyaluronate (Lee and Choi, 1999; Shasrabudhe and Deodhar, 2010). 50 μl of bovine hyaluronidase (7900 units/ml) was dissolved in 0.1 M acetate buffer (pH 3.6). Then this solution was mixed with 50 μl of different concentrations of the oils

Table 8
Inhibitory effect of the essential oils from *Pinus brutia, Pinus halepensis, Pinus nigra, Pinus pinea* and *Pinus sylvestris* on acetic acid-induced increased capillary permeability.

Material	Parts used	Dose (mg/kg)	Evans blue concentration (μg/ml) ± SEM	Inhibition (%)
Control			8.93 ± 1.17	
Pinus brutia	Cones	100	9.80 ± 0.95	–
		200	8.79 ± 0.72	1.6
	Needles	100	10.61 ± 1.56	–
		200	8.49 ± 1.43	4.9
Pinus halepensis	Cones	100	7.80 ± 0.93	12.7
		200	6.97 ± 0.91	21.9
	Needles	100	8.18 ± 1.36	8.4
		200	7.47 ± 1.22	16.3
Pinus nigra	Cones	100	8.44 ± 1.21	5.5
		200	8.14 ± 1.12	8.9
	Needles	100	7.99 ± 1.39	10.5
		200	6.17 ± 0.78	30.9*
Pinus pinea	Cones	100	7.27 ± 0.85	18.6
		200	6.27 ± 0.41	29.8**
	Needles	100	10.60 ± 1.27	–
		200	7.73 ± 1.06	13.4
Pinus sylvestris	Cones	100	8.94 ± 1.06	–
		200	9.14 ± 1.14	–
	Needles	100	7.23 ± 1.15	19.0
		200	6.46 ± 0.56	27.7*
Indomethacin		10	3.99 ± 0.21	55.3***

S.E.M.: standard error mean.
* $p < 0.05$ significant from the control.
** $p < 0.01$ significant from the control.
*** $p < 0.001$ significant from the control.

dissolved in 5% DMSO. For the control group 50 μl of 5% DMSO was added instead of the oils. After 20 min incubation at 37 °C, 50 μl of calcium chloride (12.5 mM) was added to the mixture and again incubated for 20 min at 37 °C. 250 μl sodium hyaluronate (1.2 mg/ml) was added and incubated for 40 min at 37 °C. After incubation the mixture was treated with 50 μl of 0.4 M NaOH and 100 μl of 0.2 M sodium borate and then incubated for 3 min in the boiling water bath. 1.5 ml of *p*-dimethylaminobenzaldehyde solution was added to the reaction mixture after cooling to room temperature and was incubated at 37 °C for 20 min when colour developed. The absorbance was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA).

2. Cobra (*Naja naja*) venom (Sigma–Aldrich, 155808-00-7) (100 μg in 20 μl saline) was incubated at 37 °C with 50 μg of hyaluronic acid in 250 μl 0.2 M sodium acetate buffer (pH 5.0) containing 0.15 M NaCl. The absorbance of the solutions was measured at 585 nm. Activity was expressed as *n* mol *N*-acetyl glucosamine released/min/mg protein. The effects of the essential oils on hyaluronidase activity of the venom were estimated by incubating the venom with increasing concentrations of the oils. (Reissig et al., 1955; Machiah et al., 2006).

2.3.4. Anti-inflammatory activity

2.3.4.1. Acetic acid-induced increase in capillary permeability. Effect of the test samples on the increased vascular permeability induced by acetic acid in mice was determined according to Whittle method (Whittle, 1964) with some modifications (Yesilada and Küpeli, 2007). Each test sample was administered orally to a group of 10 mice in 0.2 ml/20 g body weight. Thirty minutes after the administration, tail of each animal was injected with 0.1 ml of 4% Evans blue in saline solution (i.v.) and waited for 10 min. Then, 0.4 ml of 0.5% (v/v) AcOH was injected i.p. After 20 min. incubation, the mice were killed by dislocation of the neck, and the viscera were exposed and irrigated with distilled water, which was then poured into 10 ml volumetric flasks through glass wool. Each flask was made up to 10 ml with distilled water, 0.1 ml of 0.1 N NaOH solution was added to the flask, and the absorption of the final solution was measured at 590 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). A mixture of distilled water and 0.5% CMC (carboxymethyl cellulose) was given orally to control animals, and they were treated in the same manner as described above.

2.3.5. Statistical analysis of the data

The data on percentage anti-inflammatory and wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of $p \leq 0.05$ were considered statistically significant.

Histopathologic data were considered to be nonparametric; therefore, no statistical tests were performed.

3. Results

Wound healing and anti-inflammatory effects of the essential oils from the cones and needles of *Pinus brutia*, *Pinus halepensis*, *Pinus nigra*, *Pinus pinea* and *Pinus sylvestris* were tested in the present study. The experimental results were presented in Tables 2–8. For the activity assessment, *in vivo* linear incision and circular excision wound models were used.

In linear incision wound model, tensile strength of the treated tissue was evaluated. Tensile strength is the resistance to breaking under tension, which indicates how much the repaired tissue resists to breaking. For this purpose the newly repaired tissue was removed and tensile strength was measured (Lodhi et al., 2006). As shown in Table 2, topical application of the ointments prepared with the essential oils from the cones of *Pinus halepensis* and *Pinus pinea* onto the incised wounds demonstrated the best wound tensile strength by the highest values of 23.8% and 38.9%, respectively on day 10. On the other hand, the rest of the oils did not show any remarkable activity in this model.

Excision wound model was used to monitor wound contraction, which was calculated as percent reduction in the wound area (Saha et al., 1997). According to experimental results in circular excision wound model, essential oils obtained from the cones of *Pinus*

halepensis and *Pinus pinea* were found to have significant wound healing effect by the contraction values of 40.07% and 54.61%, respectively (Table 3). The results were in accord with the outcome of the linear incision wound model.

Treated skin samples were also assessed for their hydroxyproline content, which gives an idea of collagen concentration. Collagen is the major component of extracellular tissue. It is composed of the amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen (Kumar et al., 2006; Nayak et al., 2007). As shown in Table 4, high hydroxyproline content was determined for the tissues treated with the ointments of essential oils of *Pinus halepensis* and *Pinus pinea* cones.

Furthermore, skin samples were evaluated histopathologically. Histopathological analyses were also in accord with the results of both excision and incision experimental methods (Table 5). For demonstrating of wound healing process, representative figures (Fig. 1), which stained with HE and VG, were also added. Phases in wound healing processes with varying degree were observed within the experimental groups. The re-modeling, especially, re-epithelization were detected respectively in the reference, *Pinus pinea*, *Pinus halepensis* and *Pinus nigra* ointment treated groups. Limited wound healing processes were seen in the *Pinus brutia*, *Pinus sylvestris*, vehicle and negative control groups. As an evidence of delaying, wound associated tissue debris are still remains in the dermal tissues of the last two groups.

4. Discussion

Hyaluronic acid, a glycosaminoglycan, is one of the chief components of the extracellular matrix. It is distributed widely in connective and epithelial tissues. Hyaluronic acid plays an essential role in wound healing since it contributes significantly to cell proliferation and migration (Stern, 2004). It grows from the base of a wound and promotes migration of fibroblasts, and endothelial cells into the wound site (Chen and Abatangelo, 1999). Therefore, agents with anti-hyaluronidase activity could contribute wound healing. As shown in the Tables 6 and 7, essential oils obtained from the cones of *Pinus pinea* and *Pinus halepensis* were found to have anti-hyaluronidase activity.

For rapid healing anti-inflammatory activity is essential. Since it provides to shorten the healing period as well as for minimal pain and scar (Clark, 1991). In the present study we also aimed to evaluate the anti-inflammatory activity of the essential oils. The results indicated that the essential oils of *Pinus nigra* and *Pinus pinea* were significantly active in the Whittle Method, which is based on the inhibition of acetic acid-induced increase in capillary permeability (Table 8).

The previous studies revealed the essential oil composition of *Pinus* species. α-Pinene was found to be the main constituent of the essential oils obtained from the cones of *Pinus halepensis*, *Pinus nigra* and *Pinus slyvestris*. Limonene and β-pinene were found in higher amounts in *Pinus pinea* (Tumen et al., 2010). In our previous study on Coniferales, essential oils rich in limonene were found to have significant wound healing activity (Tumen et al., 2010). In early studies, limonene was reported to be an important monoterpene in wound healing process (Adams and Thrash, 2010). Therefore, high amount of limonene in *Pinus pinea* could promote the wound healing activity.

Essential oils obtained from the needles of *Pinus* species showed no significant wound healing effect. Gas chromatography analysis showed that limonene content of the essential oil obtained from the needles of *Pinus pinea* was comparatively high but not as high as the essential oil of *Pinus pinea* cones (Tumen et al., 2010).

Terpenic compounds were shown to have bactericidal, fungicidal, insecticidal, anticarcinogenic, pesticidal, antioxidant, anti-inflammatory, analgesic and sedative effects (Takayama et al., 2011). For wound healing activity, antibacterial agents have an important role. They provide a barrier against microbial attacks and protect the wounded area from several infections. The wound healing activity of the pine essential oils could be attributed to their antimicrobial effects.

According to experimental results, essential oils obtained from *Pinus pinea* and in *Pinus halepensis* were found to have better activity on the wound healing compared to the other oils and control groups. This study provides evidence to the folkloric use of the *Pinus* genus in wound healing.

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